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Lacrimal gland development: from signaling interactions to regenerative medicine

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Abstract

The lacrimal gland plays a pivotal role in keeping the ocular surface lubricated, and protecting it from environmental exposure and insult. Dysfunction of the lacrimal gland results in deficiency of the aqueous component of the tear film, which can cause dryness of the ocular surface, also known as the aqueous-deficient dry eye disease. Left untreated, this disease can lead to significant morbidity, including frequent eye infections, corneal ulcerations and vision loss. Current therapies do not treat the underlying deficiency of the lacrimal gland, but merely provide symptomatic relief. To develop more sustainable and physiological therapies, such as in vivo lacrimal gland regeneration or bioengineered lacrimal gland implants, a thorough understanding of lacrimal gland development at the molecular level is of paramount importance. Based on the structural and functional similarities between rodent and human eye development, extensive studies have been undertaken to investigate the signaling and transcriptional mechanisms of lacrimal gland development using mouse as a model system. In this review, we describe the current understanding of the extrinsic signaling interactions and the intrinsic transcriptional network governing lacrimal gland morphogenesis, as well as recent advances in the field of regenerative medicine aimed at treating dry eye disease.

Keywords

Lacrimal gland; dry eye; FGF; BMP; stem cell; regeneration

Overview of the lacrimal gland

The lacrimal gland is a tubulo-acinar exocrine gland that produces the aqueous component of the tear film, including water, electrolytes and proteins (Zoukhri, 2010). Critical for ocular health and quality vision, the tear film forms a smooth refractive layer over the cornea, while lubricating the cornea and conjunctiva, supporting ocular surface metabolism, and flushing away dirt and noxious stimuli. This film is composed of three layers: 1) the outermost lipid layer secreted by Meibomian glands that prevents evaporation of tears, 2) the

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middle aqueous layer produced by the lacrimal gland that accounts for over 90% of tear volume, and 3) the innermost mucous layer produced by goblet cells of the conjunctiva that anchors the tear film to the ocular surface (Johnson and Murphy, 2004). By releasing immunoglobulins into the tears, the lacrimal gland also functions as a secretory immune system to protect the ocular surface against infection (Holly and Lemp, 1977).

In humans, the primary lacrimal gland is located within the upper temporal orbit, emptying its secretions into an anastomosed duct system that delivers the fluid to the ocular surface. The outflow component of the lacrimal system lies at the nasal side of the eye, where puncta located on the upper and lower lids drain fluid into canaliculi leading to the nasolacrimal sac and nose (Figure 1) (Walcott, 1998). In rodents, however, the lacrimal gland is comprised of two lobular structures: one intra-orbital and the other extra-orbital. The primary lacrimal gland is the extra-orbital lobe, located just beneath the ear and connected to the eye via a long duct that joins the intra-orbital lobe just prior to reaching the eye (Figure 2) (Dartt, 2009). The lacrimal gland epithelium is composed of three major cell types: acinar, ductal and myoepithelial cells. The primary secretory apparatus is composed of acinar cells, which make up to 80% of the gland. The luminal sides of the acinar cells are connected to the secretory ducts lined by cuboidal duct cells, which constitute 10–12% of the lacrimal gland cell population and contribute to 30% of the lacrimal gland fluid secretions (Mircheff, 1989). Myoepithelial cells surround the basal side of both acinar and ductal cells. Their function is to apply pressure to the secretory cells to expel the fluid into the duct (Dartt, 2009). Besides these three main cell types, the lacrimal gland stroma also contains fibroblasts that produce collagens, and mast cells that secrete histamines and matrix proteins into the interstitial spaces (Walcott, 1998). The vasculature of the lacrimal gland also brings in plasma cells, lymphocytes, dendritic cells and macrophages, which provide immune protection to the ocular surface (Allansmith et al., 1976; Dartt, 2009). The function of the lacrimal system is controlled by sensory afferent nerves from the cornea and conjunctiva, coupled with parasympathetic and sympathetic efferent nerves innervating the lacrimal gland. These nerves ensure an optimum volume and quality of tear secretion in response to environmental stress (Dartt, 2009).

Impairment of lacrimal gland function can result in the debilitating condition known as aqueous-deficient dry eye disease, which can progress to corneal ulceration and vision loss if left untreated. The most significant risk factor for dry eye disease is aging, associated with structural and functional changes in the lacrimal gland characterized by atrophied acini, duct obstruction, lymphocytic infiltration and decreased protein secretion (Rocha et al., 2008). Lacrimal gland dysfunction can also arise from inflammation triggered by the dry environment, auto-immune attack as in Sjogren’s syndrome and rheumatoid arthritis, side effects of chemo and radiation therapies, as well as congenital defects (Javadi and Feizi, 2011; He et al., 2013; Park et al., 2015). Several treatments exist, including punctal occlusion to reduce tear drainage, anti-inflammatory drugs such as topical cyclosporine and lifitegrast, and most commonly artificial tears and gels (Babic et al., 2010). However, each of these clinical interventions are primarily palliative, and are not aimed at curing the underlying lacrimal gland deficiency. To this end, regeneration of the damaged lacrimal gland or replacement by bioengineered implants can potentially provide long lasting and physiological cures for dry eye disease. Ensuring the success of these approaches will

require a thorough understanding of the molecular mechanism of lacrimal gland development and regeneration. In this review, we will focus on signaling pathways and transcription factors that have been shown to regulate lacrimal gland development in animal models and discuss emerging regenerative therapies that may ultimately provide more sustainable treatment for the dry eye disease.

The molecular mechanism of lacrimal gland development

Signaling interactions: the epithelium-mesenchyme interaction

The lacrimal gland forms as a result of interactions between the conjunctival epithelium and the periocular mesenchyme. In humans, it begins as a thickening of the epithelium at the superior conjunctival fornix, which subsequently invades the underlying mesenchyme to form a highly branched gland (de la Cuadra-Blanco et al., 2003). This is recapitulated in mouse as the budding of the conjunctival epithelium at the temporal side of the eye at the E13.5 stage (Figure 2) (Makarenkova et al., 2000). This tubular bud elongates dorsally toward the ear, accompanied by condensation of the surrounding mesenchyme (Dean et al., 2004). This process can occur independently of retina and lens development, as the lacrimal gland bud develops even in mouse mutants lacking the eyeball (Swindell et al., 2008). Starting at E16.5, the lacrimal gland bud branches out to form a complex intra-orbital and extra-orbital multi-lobular structure, eventually becoming composed of a system of acini, ducts, myoepithelial cells, nerves, plasma cells and connective tissues.

The inductive signals to initiate lacrimal gland budding and branching morphogenesis are Fibroblast Growth Factors (FGFs). *Fgf10* in particular is expressed in a distinctive domain in the mesenchyme surrounding the epithelial bud and its expression persists throughout lacrimal gland development. By contrast, *Fgf7* expression in the mesenchyme is more diffused (Govindarajan et al., 2000; Makarenkova et al., 2000). Both recombinant human FGF10 and FGF7 were able to induce ectopic budding of the lacrimal gland epithelium in explant cultures of the mouse embryonic eye (Makarenkova et al., 2000). Remarkably, ectopic glands can even be induced in the cornea by transgenic expression of either rat *Fgf10* or human *FGF7* in the lens, but not by other FGFs, underscoring the potency and specificity of the FGF7/10 subfamily of FGFs for lacrimal gland development (Lovicu et al., 1999; Govindarajan et al., 2000). *Fgf10* null mice exhibit a complete loss of the epithelial component of the lacrimal gland despite an intact mesenchyme, while *Fgf7* knockout mice have normal lacrimal glands, indicating that *Fgf10* is the primary driver of lacrimal gland development (Lovicu et al., 1999; Govindarajan et al., 2000; Makarenkova et al., 2000). Further, *Fgf10* is haploinsufficient for lacrimal gland development in both mice and humans, in which a heterozygous loss of function mutation can lead to aplasia of the lacrimal and salivary glands (ALSG), a rare disorder characterized by dryness of the eye and mouth (OMIM #180920) (Entesarian et al., 2005). A more severe congenital disorder called Lacrimo-auriculo-dento-digital (LADD) syndrome affecting lacrimal and salivary glands, ears, teeth and distal limbs has been associated with missense mutations in *FGF10* (OMIM #149730) (Rohmann et al., 2006). The majority of LADD mutations result in the disruption of FGF10 protein stability or its capacity to interact with its receptor, but missense mutations affecting secretion and nuclear localization of FGF10 have also been identified (Shams et

al., 2007; Mikolajczak et al., 2016). These *FGF10* mutations are thought to exert a dominant-negative effect instead of simple loss-of-function, which may explain why more organs are affected in LADD syndrome than in ALSG syndrome (Rohmann et al., 2006). These phenotypes highlight the pivotal role of *FGF10/Fgf10* in multi-organ development, but also raise the interesting question of why lacrimal gland development is particularly sensitive to their gene dosage.

In addition to the precise control of *Fgf10* at the transcriptional level, the concentration of Fgf10 protein in the periocular mesenchyme is also under exquisite regulation by proteoglycans within the extracellular matrix (ECM) (Figure 3) (Balasubramanian and Zhang, 2016). Previous work from our lab has shown that glycosaminoglycans (GAGs) attached to proteoglycans in the periocular mesenchyme restricts the diffusion of Fgf10 during lacrimal gland development (Qu et al., 2012). Mesenchyme-specific knockouts of the proteoglycan biosynthetic enzyme UDP-Glucose 6-Dehydrogenase (Ugdh) cause excessive diffusion of Fgf10 that is found to disrupt lacrimal gland budding. Interestingly, the lacrimal gland defect can also be produced by mesenchymal specific deletion of heparan sulfate (proteoglycan) modification enzymes *N*-deacetylase/*N*-sulfotransferase (Ndst1/2), but not by 2-*O*-sulfotransferases (Hs2st) and 6-*O*-sulfotransferases (Hs6st1/2), suggesting that *N*-sulfation of heparan sulfates is essential for regulating Fgf10 compartmentalization (Qu et al., 2012). Consistent with this model, mutating the key residues of FGF10 that interact with heparan sulfates also resulted in an increased diffusion range of FGF10 in the ECM (Makarenkova et al., 2009). The mutant FGF10 was found to behave like FGF7, which has a lower affinity for heparan sulfates and preferentially promotes lacrimal gland branching instead of elongation.

FGF signaling has been shown to cooperate with the transcription factor Barx2 in the lacrimal gland epithelium to regulate the expression of two matrix metalloproteinases (MMP2 and MMP9) involved in ECM remodeling and are secreted into the mesenchyme to promote the release of Fgf10 from proteoglycans (Tsau et al., 2011). This presents a positive feedback mechanism to modulate the Fgf10 concentration ahead of the invading epithelial bud. One question that remains unresolved is whether the control of Fgf10 diffusion by proteoglycans generates a chemokine gradient to spatially guide lacrimal gland development. Answering this question is hampered by the lack of a sensitive assay to determine the endogenous concentration of Fgf10 in the periocular mesenchyme. In this regard, it is worth noting that although endogenous *Fgf10* is also expressed in a localized fashion in the embryonic lung, a recent study showed that it can be functionally substituted by ubiquitous expression of *Fgf10* during branching morphogenesis (Volckaert et al., 2013). This raises the possibility that an Fgf10 gradient may not be an absolute requirement for the budding and branching of glandular organs.

Biochemical studies have determined that the specific receptor for Fgf10 is Fgf receptor 2(III)b (Fgfr2b), which is expressed in the lacrimal gland epithelium (Makarenkova et al., 2000; Zhang et al., 2006). Indeed, both epithelial ablation of *Fgfr2* in vivo, as well as ex vivo knock down of *Fgfr2b,* disrupts lacrimal gland development (Makarenkova et al., 2000; Pan et al., 2008). Interestingly, LADD syndrome can be caused by either *FGFR2* or *FGFR3* missense mutations, both of which are assumed to be hypomorphic given that affected

patients do not exhibit the typical gain-of-function FGF signaling phenotypes (Rohmann et al., 2006). This observation is in apparent conflict with both the low affinity of Fgfr3 toward Fgf10 in vitro, and the lack of a lacrimal gland defect in *Fgfr3* knockout mice (AG and XZ, unpublished results). A functional study is needed to resolve the nature of the LADD-associated *FGFR3* mutations. It is also interesting to note that heterozygous ablation of Fgfr2c, which is not the canonical receptor for Fgf10, results in secondary branching defects in the lung, kidney and lacrimal gland (Hajihosseini et al., 2001). Since the *Fgfr2* heterozygous null mouse lacks an overt phenotype, it is believed that the *Fgfr2c* mutant is a gain-of-function allele resulting from an alternative splicing event in the *Fgfr2* locus, leading to ectopic expression and activation of Fgfr2b in the mesenchyme. Although the *Fgfr2c* mutant lacrimal gland retains a mesenchymal sac without *Fgf10* expression, it remains to be determined how aberrant Fgfr2b signaling can be activated without Fgf10 in the mesenchyme.

The assembly of an FGF signaling complex on the cell surface requires heparan sulfates as co-receptors (Figure 3). Interestingly, the lacrimal gland bud specifically expresses Ndst1 enzyme in the tip cells, but not in the follower cells that form its stalk (Pan et al., 2008). Ablation of Ndst1 in the epithelium not only disrupts *N*-sulfation of heparan sulfates, but also abrogates lacrimal gland budding. Similarly, 2-*O* and 6-*O* sulfation of heparan sulfates each contribute to Fgf10-induced signaling given that the deletions of *Hs6st* and *Hs2st* in the lacrimal gland epithelium resulted in either stunted growth or no bud formation (Qu et al., 2011). Indeed, using a FGF ligand and carbohydrate engagement assay (LACE), we showed that recombinant Fgf10/Fgfr2b proteins were able to form a tight binding complex on the lacrimal gland bud in situ, which was disrupted in heparan sulfate *N*- or *O*-sulfation mutants (Pan et al., 2008; Qu et al., 2011). On the other hand, these modifications of heparan sulfates are also under control of FGF signaling, as epithelial ablation of Fgfr2 abolished *N*-sulfation of heparan sulfates (Pan et al., 2008). This positive feedback mechanism is mediated by Shp2, a non-receptor tyrosine phosphatase that transmits FGF signaling to the Ras-MAPK pathway, partly by inhibiting the negative Ras signaling regulator Sprouty2 (Pan et al., 2010). The key targets of this FGF signaling cascade are likely to be the transcription factors Sox9 and Sox10, which have been shown to regulate the expression of heparan sulfate 3-O-sulfotransferases in an FGF-signaling-dependent manner (Chen et al., 2014b).

While Fgf10 is expressed exclusively in the lacrimal gland mesenchyme to guide budding and branching of the epithelium, another growth factor, Bmp7, displays a more complex and dynamic expression pattern during lacrimal gland development. Initially expressed in the periocular mesenchyme surrounding the epithelial bud, Bmp7 is later present in both the epithelial and mesenchymal compartments of the lacrimal gland (Dean et al., 2004). The primary target of Bmp7 signaling, however, appears to be the lacrimal gland mesenchyme. Exposing isolated lacrimal gland epithelium to Bmp7 does not affect budding induced by Fgf10, but, in a mesenchymal culture, Bmp7 resulted in increased cellular proliferation and aggregation marked by expression of connexin43, cadherins and α-smooth muscle actin (α-SMA) (Dean et al., 2004). In contrast, defective condensation of the periocular mesenchyme was found in *Bmp7* null mice, which also exhibited smaller glands with misplaced buds and reduced branching. It is thought that the condensation and proliferation of mesenchymal cells induced by BMP signaling is critical for proper branching morphogenesis of the

lacrimal gland epithelium. In support of this concept, it was shown that transcription factor Foxc1 was dispensable in the lacrimal gland epithelium, but its loss in the mesenchyme prevented BMP signaling from inducing cellular condensation (Mattiske et al., 2006). As a result, *Foxc1* null mice exhibited reduced lacrimal gland size with fewer terminal buds, reminiscent of the *Bmp7* null phenotype. In contrast to Bmp7, Bmp4 is found to suppress Fgf10-induced growth and elongation of the lacrimal gland bud in an isolated epithelial culture, suggesting that BMP signaling may also play a direct role in the lacrimal gland epithelium (Dean et al., 2004). BMP signaling is mediated by phosphorylated Smad1/5/8 proteins, which form a complex with Smad4 to activate the downstream transcriptional events. Indeed, a recent study showed that epithelial deletion of Smad4 resulted in smaller lacrimal glands with fewer branches and acini (Liu and Lin, 2014). Interestingly, the lacrimal gland in *Smad4* mutants accumulates pigments after birth and is eventually replaced by adipose tissue. These studies suggest that BMP signaling in both the epithelium and the mesenchyme are critical for lacrimal gland development.

Canonical Wnt signaling has been shown to interact with both FGF and BMP signaling to modulate lacrimal gland branching morphogenesis. Activation of the canonical Wnt signaling pathway prevents degradation of β-catenin in the cytoplasm, which is thereby translocated into the nucleus to bind Tcf/Lef transcription factors to induce gene expression. Transcripts of several Wnts (both canonical and non-canonical) are present in the lacrimal gland during development (Dean et al., 2005). Inhibition of Wnt signaling by knocking down β-catenin with morpholinos in lacrimal gland explants leads to increased branching and cell proliferation, and an up-regulation of Fgf10 in the mesenchyme. Activation of Wnt signaling by Wnt3a or LiCl treatment, on the other hand, reduces proliferation of both the epithelial and mesenchymal components of the lacrimal gland with a concurrent reduction in the number of branches. Wnt signaling also suppresses the Bmp7-induced increase of cell proliferation in the lacrimal gland mesenchyme. Thus, Wnt signaling regulates branching morphogenesis by counterbalancing the effects of Fgf10 and Bmp7 (Dean et al., 2005).

Maturation and homeostasis of the lacrimal gland also require Notch signaling, which acts through nuclear translocation of the Notch Intracellular Domain (NICD). NICD subsequently interacts with the recombination signal binding protein for immunoglobin J_k region (RBP-J_k), Histone acyl transferases, and Mastermind-like transcriptional co-activator (Maml), to activate transcription of target genes. Postnatal knockout of *Notch1* in the ocular surface causes lacrimal gland degeneration with infiltration of monocytic cells, resulting in a marked reduction in tear volume (Zhang et al., 2013). Maml-mediated Notch signaling is also responsible for maintaining the conjunctival epithelial identity and goblet cell differentiation. This is achieved by augmenting the expression of Klf4/5 transcription factors to control Muc5a expression. Indeed, lacrimal gland in mice containing a Klf5 deletion in the epithelium exhibit excessive inflammation and disorganization of the lacrimal acini (Kenchegowda et al., 2011). More recently, Notch signaling has also been proposed to regulate branching morphogenesis by suppressing cleft-formation (Dvoriantchikova et al., 2017). These studies suggest that Notch signaling contributes to both the development and function of the lacrimal gland.

In summary, after the critical role of Fgf10 in lacrimal gland development was discovered less than two decades ago, it is now appreciated that FGF signaling must interact with other pathways, including BMP, Wnt and Notch, to regulate lacrimal gland budding and branching morphogenesis. Additionally, recent high throughput gene expression analysis has also implicated IGF, TGFβ and Hippo signaling in the human lacrimal gland (Aakalu et al., 2017). In spite of these important findings, our understanding of essential molecular details remains rudimentary. For example, the lacrimal gland epithelum requires an inductive signal from the periocular mesenchyme, but how the mesenchyme itself is specified and whether the signaling is reciprocal in nature is not clear. There are also many unanswered questions regarding the mechanisms of FGF signaling itself. For example, why is Fgf10 particularly potent in inducing ectopic ocular gland, while other Fgfs, such as Fgf1, lack such activity (Robinson et al., 1995; Lovicu et al., 1999; Govindarajan et al., 2000)? This can not be entirely explained by the specificity of Fgf10 for Fgfr2b, because Fgf1 is also capable of activating the same receptor. Downstream of the Fgf receptor, there are multiple intracelullar pathways, including those mediated by Ras-MAPK, PI3K-AKT and PLC-PKC. The specific roles of each, and their downstream targets, are not yet well understood, and require further research.

Transcriptional network in lacrimal gland development

While signaling pathways transduce critical guidance information for lacrimal gland morphogenesis, transcription factors are the ultimate downstream interpreters and executors of the developmental program. The paired-domain transcription factor Pax6 is considered the master regulator of eye development (Gehring and Niimi, 1999). Its expression precedes the budding of the lacrimal gland in the fornix of the conjunctival epithelium and continues in the lacrimal gland epithelium throughout development. A loss of function mutation in even a single allele of *Pax6* results in severe impairment in mouse lacrimal gland development, suggesting that Pax6 serves as a competence factor in the epithelium (Makarenkova et al., 2000). In fact, detailed characterizations of *Pax6* enhancers have led to the development of the *Le-Cre* transgene, which can act as both a Cre deletor and reporter in the lacrimal gland epithelium, greatly facilitating the genetic analysis of its development (Ashery-Padan et al., 2000; Pan et al., 2008). Surprisingly, lacrimal gland defects have not been reported in human aniridia (OMIM 106210), a congenital disorder caused by heterozygous mutations in *PAX6*. Instead, patients with otofaciocervical syndrome-2 carrying homozygous *PAX1* mutations (OMIM 615560) display lacrimal duct abnormalities, a phenotype shared with the closely related otofaciocervical syndrome-1 (OMIM 601653) that harbors mutations in the *EYA1* gene (Pohl et al., 2013). On the other hand, branchiootorenal syndrome-1 (BOR1, OMIM #113650) caused by heterozygous *EYA1* mutations and branchiootic syndrome-3 (OMIM #608389) caused by *SIX1* heterodeficiency display many overlapping phenotypic traits including lacrimal gland stenosis, suggesting that these two genes may act in the same genetic cascade. Indeed, Six1 is found in both the duct and acini of the mouse lacrimal gland, and *Six1* knockout embryos exhibit small lacrimal glands with poor duct elongation and reduced branching (Laclef et al., 2003). From *Drosophila* to mammals, *Pax*, *Six* and *Eya* genes have been shown to form a conserved transcriptional network in organogenesis, interactions that may conceivably still occur in lacrimal gland development.

Whereas Pax/Six/Eya genes likely serve as competence factors for lacrimal gland development, additional transcription factors are required to specify the identity of the epithelium. TP63 is a transcription factor that is important for a variety of epithelial structures (Yang et al., 1999). Mutations in this gene abolish lacrimal gland development in mice, and cause Limb-mammary syndrome (OMIM 603543) associated with lacrimal-duct atresia and obstructed lacrimal puncta in humans (van Bokhoven et al., 2001). Otx1 is a homeodomain transcription factor expressed in the conjunctival epithelium and *Otx1* knockout mice fail to develop lacrimal glands (Acampora et al., 1996). In addition, loss of epithelial expression of *Runx1* results in a delay in embryonic lacrimal gland development characterized by reduced branching and a smaller lacrimal bud at E16.5. It is likely that *Runx1* is compensated in part by *Runx2* and *Runx3*, which are also expressed during the time of lacrimal gland development (Voronov et al., 2013).

Certain transcription factors may regulate development of both the epithelial and mesenchymal compartments of the lacrimal gland. The majority of patients carrying heterozygous *SOX10* mutations (Waardenburg syndrome, OMIM 611584 and 613266) have either a hypoplastic lacrimal gland or are lacking one entirely, underscoring the requirement of *SOX10* for lacrimal gland genesis (Elmaleh-Berges et al., 2013). This finding is further supported by the finding that lacrimal gland defects are present in mice harboring a conditional deletion of *Sox10* in the epithelium (Chen et al., 2014b). However, since Sox10 is also expressed by migratory neural crest cells that eventually form the periocular mesenchyme, Sox10 may also indirectly regulate lacrimal gland induction by controlling neural crest migration and differentiation. Similarly, the *TFAP2a* mutations carried by Branchiooculofacial syndrome patients cause lacrimal duct obstruction (OMIM #113620). Given that Ap2a in the mouse is expressed in both the neural crest and surface ectoderm, we predict that human TFAP2a function may be required by both the mesenchyme and epithelium of the lacrimal gland.

Our current knowledge of lacrimal gland transcription factors is largely restricted to those that are active in the epithelium rather than in the mesenchyme. This is in part due to the fact that over 90% of the mature lacrimal gland is epithelial in origin. Mesenchymal condensation is one of the earliest events in lacrimal gland development, but its mechanism and functional significance remain poorly understood. Considering the importance of the mesenchyme in inducing the development of the lacrimal gland epithelium, more efforts should be devoted to understanding its specification and differentiation in the context of lacrimal gland development. In addition, many of the human congenital syndromes mentioned earlier affect the formation of the lacrimal puncta and canaliculi, which are structural components of the lacrimal outflow system rather than being part of the parenchymal ducts existing within the gland itself. In contrast, mouse studies have revealed far more genetic mutations directly disrupting the lacrimal gland itself. It is possible that subtle structural defects in human lacrimal glands, especially those affecting the lacrimal gland duct, are under-recognized because they are both difficult to diagnose and are obscured by the general dry eye symptoms. In contrast, obstruction of the excretory duct is more clinically apparent in its presentation. Further study of lacrimal gland abnormalities in animal models is therefore important to alert and inform clinicians to the potential sources and sites of pathology in various dry eye conditions in humans.

Understanding lacrimal gland regeneration

Various secretory glandular systems including the salivary gland (Takahashi et al., 1998; Takahashi et al., 2004), pancreas (Mansouri, 2012) and mammary gland (Shackleton et al., 2006) have at least a limited ability to self-renew after injury, a process that is mediated by tissue stem cells. Similar to these exocrine glands, the lacrimal gland has also been shown to have significant regenerative potential, raising the possibility that it may be feasible to design therapeutic strategies that take advantage of this feature. Important issues to consider in this context include the molecular cues that trigger regeneration, the signaling pathways involved in the process, the nature and origin of the stem and progenitor cells, and whether these cells can be isolated and expanded in culture.

Recent studies suggest that the mature lacrimal gland can exhibit a robust regeneration program as part of the wound healing process, displaying similar features found in lacrimal gland development. This was first observed in an injury model where the pro-inflammatory cytokine IL1 was injected into the extra-orbital lacrimal gland of adult mice (Zoukhri et al., 2002; Zoukhri et al., 2007). Within 2–3 days of injection, a population of mesenchymal cells marked by the intermediate filament nestin proliferated to repair the injured gland. Interestingly, a subset of nestin-positive cells expressed α-SMA, a myoepithelial cell marker, suggesting a common origin of the acinar and myoepithelial cells involved in the repair process (Zoukhri et al., 2008). This finding is reminiscent of lacrimal gland development wherein common epithelial progenitors give rise to both acinar and myoepithelial cells. The injury and repair process was accompanied by an initial increase and subsequent decline of BMP signaling as indicated by changes in phospho-SMAD1/5/8 levels (Zoukhri et al., 2008). There was also concomitant up-regulation of *Runx* genes, which, as mentioned previously, are known to regulate lacrimal gland development(Voronov et al., 2013). These studies highlight a conservation of developmental pathways during regeneration/repair.

A critical question to consider is the identity of the stem or progenitor cells responsible for lacrimal gland regeneration. Various studies have suggested that they could be of either mesenchymal, ductal, myoepithelial or acinar origin. The nestin-positive cells isolated from IL1-injected lacrimal glands can be expanded in vitro to form adipocytes with at least some of these cells expressing the mesenchymal stem cell (MSC) markers vimentin, ABCG2, and Sca1 (You et al., 2011). However, transcription factor Snail1, which is involved in the epithelial-mesenchymal transition (EMT), is also induced in the duct epithelial cells upon injury, suggesting that the duct cells may be a source of MSCs during repair (You et al., 2012). In this regard, it is interesting to note that KRT15 is present throughout the lacrimal gland epithelium during embryonic development, but its expression in the adult lacrimal gland becomes restricted to a subset of basal cells surrounding the intercalated duct, a pattern resembling the stem cell niche reported in mammary and salivary glands (Hirayama et al., 2016). On the other hand, an immature stem-like population expressing the putative stem cell markers nestin, Mushahi1 and ABCG2 has been identified in the myoepithelium from the uninjured rat lacrimal gland. These cells can be expanded in culture and differentiated into multiple lineages, suggesting a myoepithelial origin for the intrinsic progenitor cells (Shatos et al., 2012). Single cells isolated from lacrimal gland epithelial cell

cultures have also been shown to possess sphere-forming capabilities characteristic of stem cells, but these cells did not express the myoepithelial marker α-SMA (Kobayashi et al., 2012). Using cell surface markers (c-kit^{+}Epcam^{-}CD31^{-}CD45^{-}Sca1^{-}), epithelial progenitor cells have been purified by FACS and shown to form organoids that differentiate into ductal and secretory cells in a 3D culture system (Gromova et al., 2017). Finally, presumptive stem cells expressing the pluripotency markers Sox2, Nanog, and Klf4 can be isolated from the adult mouse lacrimal gland, passaged multiple times in culture, and induced to express markers of all three germ layers (Ackermann et al., 2015). In the human lacrimal gland, stem-cell like cells positive for ABCG2, ALDH and c-kit have also been reported to form spheres in non-adherent cultures.

Taken together, the emerging consensus is that the adult lacrimal gland harbors endogenous stem or progenitor cells, but their identity and location remains controversial. Because the existing studies are limited to in vitro cultures and putative stem cell markers, they may not accurately characterize lacrimal gland stem cells in vivo. We suggest that studies of lacrimal gland regeneration would benefit from the genetic approaches that have propelled the studies of lacrimal gland development. We have previously used the *Le-Cre* driver to trace the lineage of the Pax6-expressing cells during lacrimal gland development, showing that they specifically reside in the lacrimal gland epithelium in new born mice (Pan et al., 2008). With the increasing repertoire of inducible Cre lines, similar lineage tracing techniques should be readily applicable in resolving the location and nature of the lacrimal gland stem cells.

Current advances in regenerative therapy for the dry eye disease

Given the lack of curative treatments for dry eye disease, regenerative medicine has emerged as a promising approach to provide more permanent and sustainable treatment options. For the treatment of dry eye disease, our understanding of developmental biology, stem cell biology, and the regenerative capacity of the lacrimal gland is of critical importance for advancing this field of medicine. Several studies have already indicated that resident stem or progenitor cells in the mature lacrimal gland can turn on the same signaling pathways and transcription factors used in embryonic development to drive proliferation and differentiation in the adult gland. Currently, two main strategies are being developed for lacrimal gland repair and regeneration: i) capitalizing on the intrinsic regenerative capacity of the lacrimal gland, and ii) developing bioengineered lacrimal glands for tissue replacement. Both strategies will require a detailed knowledge and understanding of lacrimal gland development and remodeling.

Promoting the intrinsic repair and regeneration of the lacrimal gland

Despite the uncertainty regarding the stem/progenitor cells in the adult lacrimal gland, there are significant efforts to develop pharmacological approaches that promote the intrinsic repair process of the gland. It has been shown that inflammation-induced injury causes apoptosis and autophagy, which then trigger the intrinsic repair process (Zoukhri, 2010). This has led to the idea that activating apoptosis through TNF-α agonists can potentially initiate in vivo regeneration of the injured lacrimal gland. In fact, the TNF-α-induced factor TSG-6 has been identified as the main cytokine responsible for the regenerative and immunomodulatory effects of mesenchymal stem cells in different tissues (Dietrich et al.,

2016). Recently, topical administration of TSG-6 has been shown to improve tear production and ameliorate ocular surface defects in a mouse model of dry eye (Lee et al., 2015a). Similarly, injection of platelet-rich activated plasma into an adjacent region of the lacrimal gland has been reported to be safe and effective in promoting tear production in Sjogren's syndrome patients, an effect that may be due to growth factors and cytokines present in the plasma (Avila, 2014). In this regard, it is notable that exogenous FGF2 has been shown to stimulate the repair of atrophic salivary glands in rats, and administration of BMP7 can preserve renal tubular function after acute and chronic kidney injury (Okazaki et al., 2000; Simic and Vukicevic, 2005). With the increasing understanding of FGF and BMP pathways in lacrimal gland development, their ligands and agonists can potentially be exploited to initiate or accelerate repair.

In addition to drug-based therapy, direct cell transplantation has also been explored. Mesenchymal stem cells (MSCs) derived from different sources, including bone marrow, adipose tissue, and glandular organs, are multipotent, non-immunogenic, and anti-inflammatory (Dietrich et al., 2016). These properties are particularly attractive in conditions such as Sjogren's syndrome, in which inflammation causes a significant decline in the functional capacity and regenerative potential of lacrimal gland progenitor cells (Umazume et al., 2015). There are several lines of evidence demonstrating the beneficial effects of administering MSC-like cells into the lacrimal gland. In a dry eye mouse model, periorbital injection of human MSCs led to a localized reduction in inflammatory cytokines and improved tear secretion (Lee et al., 2015b). Topical administration of MSCs in a rat model of the dry eye disease also improved tear volume and tear film stability (Beyazyildiz et al., 2014). Recently, progenitor cells of the epithelial lineage from adult mouse lacrimal glands were successfully engrafted into the ductal and acinar compartments of chronically diseased lacrimal gland in $TSP^{-/-}$ mice, a model of Sjogren's syndrome. This procedure partially restored the structural integrity of the lacrimal gland and improved tear secretion (Gromova et al., 2017). These results suggest that cell-based therapy is a promising approach for treating the aqueous-deficient dry eye diseases.

Developing bioengineered lacrimal gland for transplantation

In the case of extensive lacrimal gland damage, direct transplantation of a donor or bioengineered lacrimal gland may be the only viable approach. However, when considering these forms of therapy, graft rejection and scant supply of donors are frequent issues that arise (Abouna, 2008). A recent study has evaluated the feasibility of porcine lacrimal gland as a potential xenograft candidate for transplantation, identifying significant similarities in morphology, anatomical location and connection to vascular supply. Before being a more commonly accepted route of therapy, however, key challenges, which include immune rejection and differences in tear composition, will need to be resolved (Henker et al., 2013). For a bioengineered lacrimal gland, advances in stem cell biology and biomedical engineering have made it increasingly promising to consider generating replacement tissues in vitro. Its ultimate success, however, requires advances in two essential areas- i) creating an abundant source of lacrimal gland cells with intact secretory function, and ii) a biocompatible scaffold to provide the appropriate microenvironment and structural support for proper histogenesis.

Sources of lacrimal gland cells—As a proof of principal, lacrimal gland germs have recently been reconstituted by combining mouse embryonic epithelial and mesenchymal cells in a gel matrix system (Hirayama et al., 2013). After engraftment into adult mice lacking the extra-orbital gland, these bioengineered germs grew and successfully restored lacrimation. Although these findings are highly promising, the use of embryonic tissue in a clinical setting is presently a grey area. Alternatively, the presence of adult lacrimal stem or progenitor cells may could be a feasible approach to reconstituting the lacrimal gland, if graft immune compatibility can be ensured.

The use of autologous induced pluripotent stem (IPS) cells can theoretically provide an unlimited supply of lacrimal gland cells without immunological complications. By providing appropriate growth factors and substrates that closely mimic the relevant environment during embryonic development, IPS cells have been successfully coaxed to differentiate into numerous cell types, including those constituting glandular organs such as the liver and pancreas (Pagliuca et al., 2014; Ishikawa et al., 2015; Pellegrini et al., 2015; Toyoda et al., 2015). A similar strategy to that used for human embryonic stem cells (hESCs) (Lee et al., 2007; Dincer et al., 2013; Leung et al., 2013) can be applied to IPS cells to derive lacrimal gland tissues. Based on data derived from the study of lacrimal gland development, we suggest that IPS cells can be induced to differentiate into 1) ocular ectodermal cells that will form precursors of the lacrimal gland epithelium, and 2) neural crest cells that will form the periocular lacrimal gland mesenchyme. Signaling molecules such as FGF10 and BMP7 can be used to promote branching morphogenesis of the epithelial cells and condensation of the mesenchymal cells, respectively. These processes can be monitored by the expression of PAX6 and OTX1 for the epithelium, and FOXC1 for the mesenchyme. Alternatively, direct conversion of the IPS cells could potentially be achieved by the forced expression of transcription factors that play important roles in lacrimal gland development. Indeed, it has been recently reported that overexpression of PAX6, FOXC1 and SIX1 induce hESCs to take on morphological and gene expression profiles that resemble the lacrimal gland epithelium (Hirayama et al., 2017). Although many technical hurdles remain, this study suggests that IPS cells may ultimately be programmable to differentiate into lacrimal gland cells.

The next challenge in establishing a reliable source of lacrimal gland cells is to expand them in culture and validate their cellular characteristics. Studies on primary acinar cells have shown that serum-free medium that contains an appropriate mixture of hormonal and growth factor supplements can preserve the cells' differentiated morphology and secretory capacity in the presence of matrigel. Nonetheless, it should be noted that reduced cell proliferation was observed (Oliver et al., 1987; Hann et al., 1989; Hann et al., 1991). To optimize culture conditions, lacrimal gland epithelial cells from newborn mice were tested for growth factor responsiveness. Both epidermal growth factor (EGF) and hepatocyte growth factor (HGF) were found to promote cell viability and proliferation. Counter-intuitively, however, FGF10 failed to produce any effect (Ueda et al., 2009). Another study, using rabbit lacrimal gland, showed that a combination of EGF, dihydrotestosterone (DHT) and matrigel was able to induce massive proliferation of acinar cells in serum-free Hepato Stim™ Medium (HSM) (Schonthal et al., 2000). These studies highlight the importance of medium formulations and substrate conditions for expanding and preserving functional lacrimal gland cells.

Building the functional lacrimal gland—To produce lacrimal gland germ for surgical implantation, lacrimal gland cells need to be seeded on an appropriate substrate that supports their structure and function. Human amniotic membranes have been shown to promote rabbit lacrimal gland cells to form acini-like structures, but their secretory response diminished over time, possibly as a result of structural impairments contained within the membranes (Schrader et al., 2007). Studies have also described the reconstitution of mouse, rat, rabbit and human lacrimal gland epithelial cells on Matrigel to form cell aggregates, displaying the morphology and secretory function characteristic of lacrimal gland acini (Meneray et al., 1994; Yoshino et al., 1995; Vanaken et al., 1998; Schechter et al., 2002; Ueda et al., 2009; Tiwari et al., 2012). However, because it is derived from mouse sarcoma tissue, there are obvious safety concerns associated with the use of Matrigel in a clinical setting (Kibbey, 1994). Several polymer-based substrates have also been tested or their acting as a scaffold in lacrimal gland morphogenesis with varying degrees of success being achieved (Long et al., 2006; Selvam et al., 2007a; Selvam et al., 2007b; Selvam et al., 2009). Long-term functional studies will be needed to evaluate the efficacy and stability of the engineered tissues.

Human lacrimal gland is organized into a three dimensional structure that is well adapted for its secretory function. To more closely mimic this configuration, lacrimal gland acinar cells from rabbit have been cultured in a microgravity bioreactor to form lacrimal spheroids. However, following a two week period, continuous cell death was frequently displayed (Schrader et al., 2009). The same group recently described a non-immunogenic decellularized tissue matrix derived from pig lacrimal gland, which allows lacrimal gland epithelial cells seeded on this scaffold to maintain acinar-like structures and secretory ability for up to 30 days (Spaniol et al., 2015). In a similar effort, rabbit lacrimal gland progenitor cells derived from a sphere-forming culture have been shown to differentiate and display secretory function in either a decellularized lacrimal gland matrix or 3-D collagen gel (Lin et al., 2016). There are still challenges regarding optimization of the decellularization technique, the seeding process, the long-term culturing conditions, and functional validation. It is notable, however, that a lacrimal outflow duct constructed using acellular bovine dermal matrix has been successfully transplanted into patients to relieve obstructive signs and symptoms (Chen et al., 2014a). Through providing effective mechanical strength, the appropriate micro-environment for lacrimal gland cells, and the necessary structural support for vascular, nerve and ductal supply, the decellularized matrix promises to be an ideal scaffold for bioengineered lacrimal gland.

Summary and future research

Aqueous-deficient dry eye disease is a major health challenge that lacks effective treatment. Although lacrimal gland transplantation is a potentially promising course of treatment, it is plagued by immunological complications and donor shortage. Development of fully functional bioengineered lacrimal glands in vitro is a viable approach to repair damaged lacrimal glands, while stimulation of the intrinsic regenerative potential of the lacrimal gland also holds great promise. Both therapeutic strategies require a deeper understanding of development and regeneration of the gland. From the development perspective, we still have several unanswered questions: 1) how do the common epithelial progenitor cells gives rise to the acinar, ductal and myoepithelial cells, 2) how is the lacrimal gland mesenchyme

specified from the neural crest cells, and 3) what factors are required for adult lacrimal gland homeostasis? Additional work is also needed to reveal the signaling cascades that underlie gland morphogenesis, and the transcriptional network that ultimately determines the tissue identity and cell lineage for repair and regeneration. Those studies will pave the way to realizing the full potential of regenerative medicine to treat dry eye disease.

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Key Findings

1. Abnormalities of the lacrimal gland underlie aqueous-deficient dry eye disease.
2. FGF signaling is the key regulator of lacrimal gland morphogenesis.
3. The adult lacrimal gland harbors intrinsic stem or progenitor cells.
4. Developmental mechanisms can guide efforts in lacrimal gland regeneration.

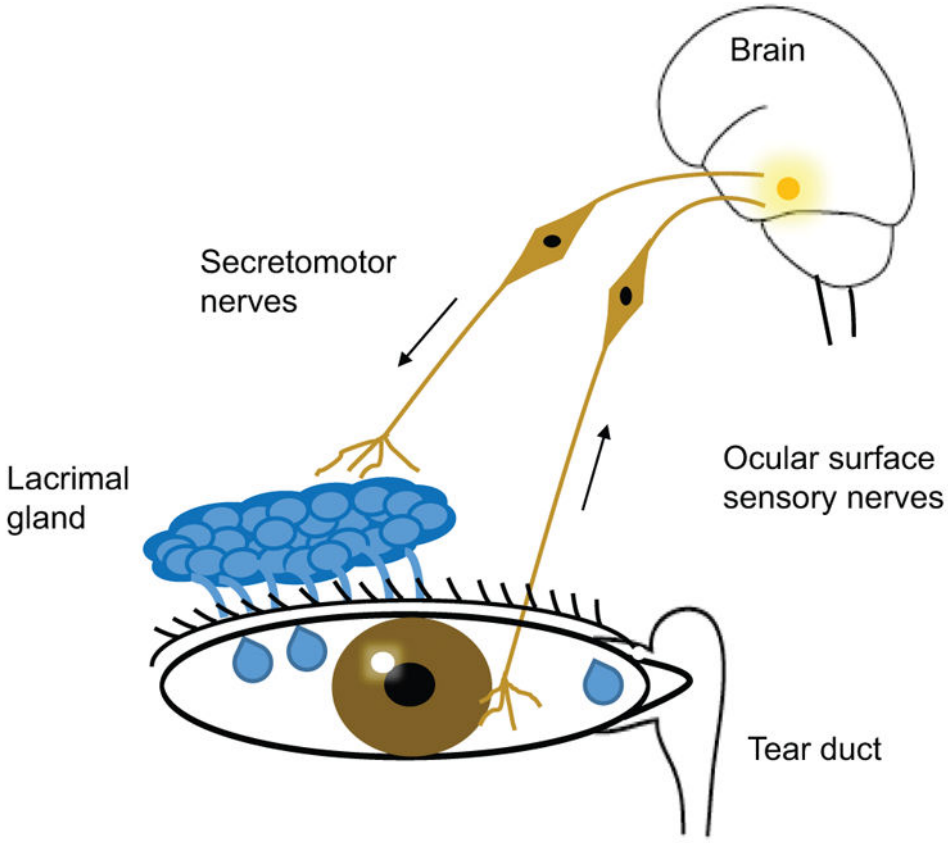

Figure 1. Schematic of Lacrimal gland functional unit

The lacrimal gland functional unit is comprised of a) the lacrimal gland, b) Sensory afferent nerves from the cornea and conjunctiva, c) motor efferent nerves originating from the central nervous system which innervate lacrimal gland, d) the excretory tear duct for drainage of the excess fluid. Impairment in any components of lacrimal gland function unit can destabilize the tear film and cause the dry eye disease.

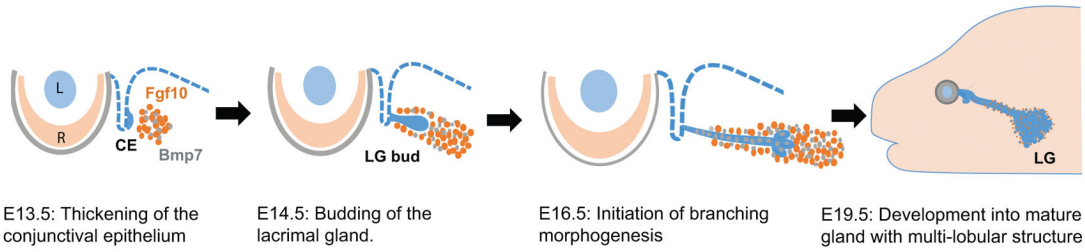

Figure 2. Lacrimal gland development in mouse

Transverse sections of mouse embryos at different stages are shown. Lacrimal gland development begins with thickening of the CE at E13.5 induced by Fgf10 from the surrounding mesenchyme. These epithelial cells further grow and elongate into a bud from E14.5 through E15.5. Branching of LG initiates at E16.5 under the additional influence of BMP7 signaling, eventually forming a multi-lobular tubulo-acinar structure at E19.5. Lacrimal gland continues to develop even during post-natal stages to become a mature gland capable of regulated tear secretion in adults. L: lens, R: Retina, CE: conjunctival epithelium, LG: lacrimal gland.

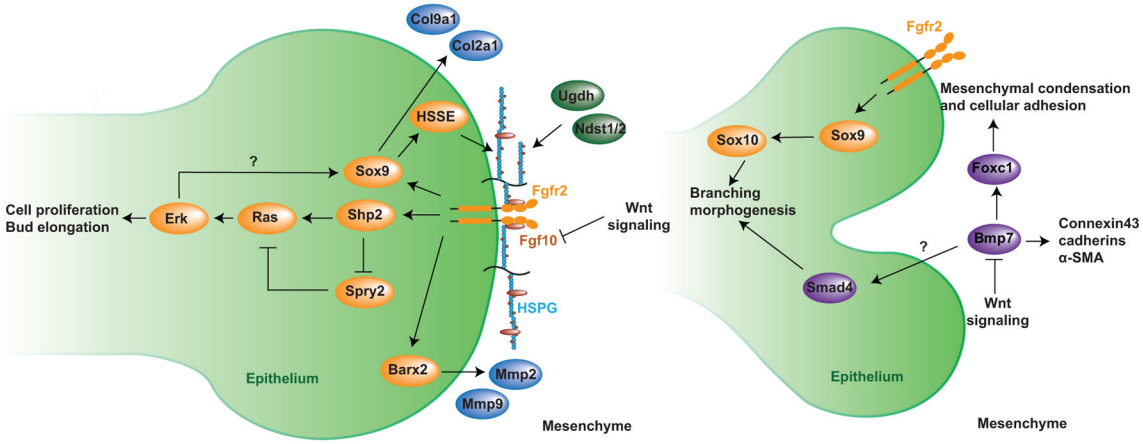

Figure 3. Summary of signaling interactions during lacrimal gland morphogenesis

(**Left**) Fgf10 forms a heparan sulfates (HS)-dependent gradient in the periocular mesenchyme, inducing lacrimal gland budding by binding to both Fgfr2b and HS in the epithelium. This activates Shp2, which inhibits the Ras signaling repressor Spry2 and promotes Ras-Erk cascade to stimulate cell proliferation, survival and bud elongation. With transcription factors Sox9 and Barx2, FGF signaling also stimulates expressions of HS synthesizing enzymes (HSSE) and metalloproteinases to remodel the ECM, forming a positive feedback loop to enhance FGF signaling activity. (**Right**) Bmp7 signaling mediated by Foxc1 is important for mesenchymal condensation during branching morphogenesis. Both FGF and BMP signaling are counterbalanced by canonical Wnt signaling in the mesenchyme. In addition, Smad4-mediated BMP signaling and Sox9-Sox10 cascade also directly regulates the epithelial elongation.

Table 1

Transcription factors implicated during lacrimal gland development and maintenance.

	Functional relevance during Lacrimal gland development	
Genes	**Mice**	**Phenotype in humans**
Pax6	Budding, competence factor	Not reported
Pax1	Not studied	Lacrimal duct stenosis
Eya1	Not studied	Lacrimal gland aplasia, duct stenosis
Six1	Branching morphogenesis	Lacrimal duct stenosis
Otx1	Budding	Not reported
p63	Budding	Lacrimal-duct atresia, obstructed lacrimal puncta
Runx1–3	Branching morphogenesis	Not reported
Klf5	Preservation of glandular function during post-natal stages	Not reported
Sox10	Growth and branching morphogenesis	Hypo-plastic or no lacrimal gland
Ap2α	Not studied	Lacrimal duct obstruction